

Title: SerpinB1 is critical for neutrophil survival through cell autonomous inhibition of cathepsin G

Running title: serpinB1 shields PMNs against CG-mediated death

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Abstract

The bone marrow (BM) holds a large reserve of polymorphonuclear neutrophils (PMNs) that are rapidly mobilized to the circulation and tissues in response to danger signals. SerpinB1 is a potent inhibitor of neutrophil serine proteases (NSPs) neutrophil elastase (NE) and cathepsin G (CG). *SerpinB1*-deficiency (*sB1*^{-/-}) results in a severe reduction of the BM PMN reserve and failure to clear bacterial infection. Using BM chimera, we found that serpinB1 deficiency in BM cells was necessary and sufficient to reproduce the BM neutropenia of *sB1*^{-/-} mice. Moreover, we showed that genetic deletion of CG, but not NE, fully rescued the BM neutropenia in *sB1*^{-/-} mice. In mixed BM chimera and in vitro survival studies, we showed that CG modulates *sB1*^{-/-} PMN survival through a cell-intrinsic pathway. In addition, membrane permeabilization by lysosomotropic agent LLME that allows cytosolic release of granule contents was sufficient to induce rapid PMN death through a CG-dependent pathway. CG-mediated PMN cytotoxicity was only partly blocked by caspase inhibition suggesting that CG cleaves a distinct set of targets during apoptosis. In conclusion, we have unveiled a new cytotoxic function for the serine protease CG and showed that serpinB1 is critical for maintaining PMN survival by antagonizing intracellular CG activity.

Introduction

Polymorphonuclear neutrophil granulocytes (PMNs) are essential components of the innate immune response to infection. PMNs are relatively short-lived leukocytes that originate from hematopoietic stem cells in the bone marrow (BM) in a process called granulopoiesis. Granulopoiesis proceeds through a proliferative phase followed by a maturation phase. After maturation, the BM retains a large reserve of mature PMNs, which includes over 90% of the mature PMNs in the body while only a small proportion (1-5%) is in the blood^{1,2}. Even in non-inflammatory conditions, granulopoiesis is remarkable as more than 10^{11} PMNs are produced daily in an adult human only to be disposed of, largely unused, a few hours later³. There is evidence that the majority of PMNs produced never reaches the circulation and die within the BM⁴. Congenital or acquired forms of neutropenia are associated with the highest risks of bacterial and fungal infection⁵, indicating a strong evolutionary pressure to maintain granulopoiesis at high levels and sustain a large mobilizable pool of PMNs in the BM.

In steady state, PMNs die by apoptosis, a form of programmed cell death that allows for the safe disposal of aging PMNs and their potentially toxic cargo. Like in other cells, caspases participate in the initiation, amplification and execution steps of apoptosis in PMNs^{6,7}. Interestingly, non-caspase cysteine proteases calpain and cathepsin D were reported to induce PMN apoptosis through activation of caspases⁸⁻¹¹. In addition, PMNs carry a unique set of serine proteases (NSPs) including elastase (NE), cathepsin G (CG) and proteinase-3 (PR3) stored active in primary granules. There is strong evidence for a role of NSPs in killing pathogens and inducing tissue injury when released extracellularly¹²⁻¹⁴. In contrast, the function of NSPs in PMN homeostasis and cell death remains elusive. In particular, no defects in granulopoiesis or PMN homeostasis have been reported in mice deficient in cathepsin G (*CG*^{-/-})¹⁵, neutrophil elastase (*NE*^{-/-})^{16,17} or dipeptidylpeptidase I (*DPPI*^{-/-}), which lack active NSPs¹⁸. We have recently shown that mice lacking the serine protease inhibitor serpinB1 (*sB1*^{-/-}) have reduced PMN survival in the lungs following *Pseudomonas* infection and that these mice have a profound reduction in mature PMN numbers in the BM^{19,20}. SerpinB1, also known as MNEI (monocyte neutrophil elastase inhibitor), is expressed at high levels in the cytoplasm of PMNs and is one of the most potent inhibitors of NE, CG and PR-3^{21,22}. In this study, we tested the hypothesis that serpinB1 promotes PMN survival by inhibiting one or several NSPs and we discovered a novel regulatory pathway in PMN homeostasis in vivo.

Methods

Mice. Generation of *serpinB1*^{-/-} (*serpinb1a*^{tm1.1Cben}) mice in 129S6/SvEvTac (129S6) and backcrossing in C57BL/6J background were described previously^{19,20}. Mice deficient in cathepsin G (CG^{-/-}) (*Ctsg*^{tm1Ley})¹⁵ and dipeptidylpeptidase I (DPPI^{-/-}) (*Ctsc*^{tm1Ley})²³ were provided by Christine Pham (Washington University, St Louis, MO). Neutrophil elastase deficient mice (NE^{-/-})(B6J-Elane^{tm1Sds})²⁴, green fluorescent protein (GFP⁺) transgenic mice (B6-Tg(CAG-EGFP)^{1Osb}) and CD45.1 (B6.SJL-*Ptprc*^a*Pepc*^b/BoyJ) were obtained from the Jackson Laboratories. All animal studies were approved by the Cantonal Veterinary Office of Bern and conducted in accordance with the Swiss federal legislation on animal welfare.

Flow cytometry and cell sorting. Leukocyte counts and hematopoietic lineage differential analysis of blood and bone marrow was performed in 8-week old mice. Equal numbers of males and females were included for each line. Blood (70µl) was collected from the retroorbital sinus of sedated mice using heparinized microhematocrit tubes. Total blood leukocyte, erythrocyte and platelet counts were performed at the central clinical laboratories of the veterinary faculty of the University of Bern. BM cells were harvested from femurs by flushing with cation-free HBSS supplemented with 1% FCS and counted in a Neubauer chamber. Isolated BM cells and whole blood were stained with fluorescently labeled antibodies (Biolegend and BD Biosciences) and analyzed on a FACScalibur flow cytometer (BD Biosciences) as described previously²⁵. Briefly, leukocyte subsets percentages were determined within CD45⁺ cells as PMNs (CD11b⁺Ly-6G^{hi}), myelocytes (CD11b⁺Ly-6G^{neg}SSC^{hi}CD115^{neg}), monocytes (CD115⁺) and B cells (CD19⁺ or CD45RB220⁺). Data was analyzed and presented using FlowJo (TreeStar). Flow sorting of PMNs, B cells and myelocytes was performed on single cell suspensions of BM leukocytes using a FACS Aria II sorter (BD Biosciences) at the flow cytometry core facility of the Department of Clinical Research of the University of Bern.

Bone marrow chimera. For the generation of chimeric mice, recipient mice were lethally irradiated from a cesium source with 10 Gray using a Gammacell 40 Exactor (Theratronics). BM cells (10-20x10⁶) from 8-13 week-old donor mice were injected into the retroorbital sinus of recipient mice 18 hr after irradiation. Antibiotics (1% Cotrim, Spirig Pharma AG) were added to the drinking water for 4 weeks and BM and blood populations were analyzed at least 8 weeks after BM transfer. Reconstitution efficiency was always greater than 95% based on flow cytometry analysis of recipient cells (CD45.1, CD45.2 or GFP). In mixed BM chimera, the relative percentage of WT (CD45.1) and *sB1*^{-/-} (CD45.2) cell subsets were

determined as described in Suppl. Fig.S3. Since B cell numbers were not different in WT and *sB1*^{-/-} mice and engrafted with a similar efficiency, B cells provided an internal control population to estimate the actual percentage of hematopoietic stem cell input of each genotype (% input) transplanted in each GFP mouse.

Western blot. Sorted cells were washed and lysed (10⁷/ml) in RIPA buffer with protease inhibitor cocktail (Roche). Lysates were resolved by SDS-PAGE under reducing conditions and immunoblotted using rabbit antiserum to serpinB1²⁰. Blots were stripped and restained with anti-β-actin antibody (Cell Signaling Technology).

In vitro survival assays. BM cells were isolated by flushing femurs and tibias of 8-22 week-old mice with HBSS. PMNs were enriched using a magnetic negative selection kit (EasySep, StemCell Technologies). Purity and viability of the purified neutrophils were assessed by flow cytometry and were typically 70-95% and >90%, respectively. Purified neutrophils were cultured in DMEM (4mM L-Glut, 25mM D-Glucose, 1mM Sodium Pyruvate)(GIBCO) containing 1% FCS at 0.5-1x10⁶cells/ml in the presence or absence of the pan-caspase inhibitor Q-VD-OPh (SM Biochemicals LLC), CG inhibitor I (JNJ-10311795; RWJ-355871)(Merck Chemicals) or L-leucyl-L-leucine methyl ester (LLME)(G-2550; Bachem). Cytotoxic effects of human CG (Athens Research & Technology) were evaluated in serum-free medium. Effects of LLME were investigated using whole BM after erythrocyte lysis by NH₄Cl. At the indicated time-points, cells were harvested and viability was assessed using AnnexinV-FITC and 7AAD. After gating on Ly-6G⁺ events, viable cells were identified as FSC^{hi}7AAD^{neg}AnnexinV^{neg}, early apoptotic as FSC^{hi}7AAD^{neg}AnnexinV⁺ and late apoptotic/necrotic as 7AAD⁺, respectively (Suppl. Fig.S1).

In vivo administration of G-CSF. Mice received daily intraperitoneal injections of recombinant murine G-CSF (500ng/mouse)(Invitrogen) or control saline for 5 days. Blood and BM were harvested 24hr after the last injection and analyzed as described above.

Statistical analysis. All analyses were performed using GraphPad Prism Mac 4.0c (GraphPad, San Diego, CA). P<0.05 was considered statistically significant.

Results

SerpinB1 neutropenia is rescued by BM transfer

We have previously shown that serpinB1 is expressed at highest levels in the granulocyte lineage and that *sB1*^{-/-} mice have a profound reduction in BM mature PMN numbers despite normal granulopoiesis¹⁹. Because serpinB1 is also expressed

in many tissues and cell types^{21,26}, we generated BM chimera to investigate the contribution of non-hematopoietic cells in sustaining PMN survival. WT mice were lethally irradiated and were transferred with WT or *sB1*^{-/-} BM cells. Eight to ten weeks after BM transfer, mice reconstituted with *sB1*^{-/-} cells recapitulated the defective PMN reserve of *sB1*^{-/-} mice, whereas the mice reconstituted with WT cells had normal numbers of BM PMNs, indicating that serpinB1-deficiency in BM cells is sufficient to induce BM neutropenia *in vivo* (Fig.1A). In the reverse experiment, transfer of WT BM cells in irradiated *sB1*^{-/-} mice restored a normal PMN reserve in the BM (Fig.1B). Overall, these results demonstrate that serpinB1-deficiency in the BM compartment is necessary and sufficient to reproduce the neutropenic defect of *sB1*^{-/-} mice.

Cathepsin G regulates neutrophil numbers in the bone marrow

Since serpinB1 is an efficient inhibitor of NE, CG and PR3, we then examined PMN numbers in mice deficient in one or several NSPs in combination with serpinB1 deletion. As expected, *sB1*^{-/-} mice had significantly reduced numbers and percentage of mature PMNs in the BM compared to WT and heterozygous *sB1*^{+/-} mice. In addition, PMN numbers were normal in mice deficient in either DPPI, NE or CG (Fig.2A,B). Dipeptidyl peptidase I (DPPI) is not inhibited by serpinB1 but is required for the activation of all NSPs and no NSP activity is detectable in *DPPI*^{-/-} mice^{18,23}. PMN counts in *DPPI*^{-/-}.*sB1*^{-/-} BM were significantly higher than in *sB1*^{-/-} BM, suggesting that one or several NSPs contribute to the PMN survival defect. To examine the role of NSPs in this process, we crossed several NSP-deficient strains with *sB1*^{-/-} mice. We found that NE.CG.*sB1*^{-/-} mice had normal PMN numbers indicating that these NSPs play a key role in the defective phenotype of *sB1*^{-/-} PMNs (Fig. 2A,B). Furthermore CG.*sB1*^{-/-} mice showed normal PMN numbers whereas NE.*sB1*^{-/-} mice retained the BM neutropenia phenotype indicating that CG, but not NE, plays a significant role in the death of *sB1*^{-/-} PMNs (Fig. 2A,B). In addition, the double-deficient *NE.sB1*^{-/-} mice had significantly lower BM myelocyte numbers than *sB1*^{-/-} mice while the myelocyte numbers in singly-deficient *NE*^{-/-} and *sB1*^{-/-} BM were normal (Fig.2C,D). These results suggest that NE may promote myeloid cell proliferation, an activity that is revealed only when serpinB1 is absent. This complex interaction between sB1 and NE requires further investigation. On the other hand, B cell and monocyte numbers and relative percentage in the BM were largely similar in all genotypes (Suppl. Fig.S2). Total numbers of blood leukocytes, erythrocytes and platelets were normal in mice deficient in NSPs and/or serpinB1 (Suppl. Fig.S3). PMN numbers in blood were normal in *sB1*^{-/-} mice in steady state and combined deficiency of NSPs did not significantly alter these numbers (Fig.2E,F). Taken

together, our results indicate that serpinB1 likely sustains the survival of post-mitotic PMNs through its interaction with CG.

CG-mediated PMN death is cell-intrinsic

We next generated mixed BM chimeras to measure PMN competitive survival as well as evaluating the potential by-stander effects of CG or serpinB1 released in the BM environment on the overall PMN survival in vivo. First, lethally irradiated GFP⁺ mice were reconstituted with a 1:1 mixture of WT (CD45.1) and *sB1*^{-/-} (CD45.2) BM cells and analyzed 8-10 weeks after reconstitution. Recipient GFP⁺ cells were always <3% and excluded from the analysis (Suppl. Fig.S4). B cells of WT and *sB1*^{-/-} origin were found in similar proportions in the BM, reflecting similar input of hematopoietic stem cells from the two donor genotypes. In contrast, the proportion of WT PMNs was significantly greater than the proportion of *sB1*^{-/-} PMNs, indicating a robust survival advantage of the WT PMNs and negligible by-stander effect of WT cells on *sB1*^{-/-} PMN survival (Fig.3A). Second, we transferred irradiated GFP⁺ mice with skewed ratios of WT and *sB1*^{-/-} BM. We found that total PMN numbers in the BM negatively correlated with the percentage of *sB1*^{-/-} input (reflected by the percentage of B cells of *sB1*^{-/-} origin): when *sB1*^{-/-} input was low, total PMN counts were similar to those of WT mice; conversely, when *sB1*^{-/-} input was high, PMN numbers corresponded to those of *sB1*^{-/-} mice (Fig.3B). Third, we found no survival defect of CG.*sB1*^{-/-} PMNs compared to WT PMNs in vivo when reconstituted at equal ratios into GFP⁺ mice (Fig.3C). Finally, isolated BM PMNs were incubated in vitro with various concentrations of human CG. Extracellular CG failed to induce a significant increase in *sB1*^{-/-} PMN death in vitro compared to WT PMNs (Fig.3D). Taken together, these results suggest that CG-mediated death in *sB1*^{-/-} PMNs is cell intrinsic.

CG-mediated PMN death proceeds independently of caspase activity

Neutrophil apoptosis whether triggered by intrinsic or extrinsic pathways culminates in the activation of effector caspases^{6,7}. We previously reported that *sB1*^{-/-} PMNs isolated from the BM show increased spontaneous apoptosis and necrosis compared to WT PMNs after 16hr ex vivo^{19,20}. To investigate whether increased *sB1*^{-/-} PMN death was dependent on caspase activity, purified BM neutrophils were cultured in the presence of the pan-caspase inhibitor Q-VD-OPh²⁷. In the absence of Q-VD-OPh, we found no significant difference in the survival of WT and *sB1*^{-/-} PMNs (survival of 31% at 24hr; 5% at 48hr; <1% at 72hr in both genotypes). Caspase inhibition significantly extended the survival of both WT and *sB1*^{-/-} PMNs compared to untreated PMNs. However, caspase inhibition was significantly less effective in

increasing *sB1*^{-/-} PMN survival compared to WT PMNs, with 32% vs 81% survival at 72hr, respectively (Fig.4A). We then attempted to block CG activity using the cell permeable CG inhibitor I²⁸. Although CG inhibitor I at 10 and 30μM led to a small but significant increase in survival of *sB1*^{-/-} PMNs, we observed simultaneous toxicity on WT PMN survival (Suppl Fig.S5) thus precluding further increase in inhibitor concentration. Since granule levels of CG are high in PMNs, sufficient concentrations of CG inhibitor may not have been reached to fully block CG activity. Therefore, we investigated whether genetic deletion of CG would affect *sB1*^{-/-} PMN survival. We found that in the doubly-deficient *CG.sB1*^{-/-} mice the absence of CG completely rescued the *sB1*^{-/-} PMN survival defect, even in the presence of Q-VD-OPh (Fig.4B). Taken together, our results unveil a CG-dependent but caspase-independent death pathway in PMNs that is controlled by serpinB1.

Granule membrane permeabilization induces CG-mediated death in PMNs

To test whether granule disruption contributes to the serpinB1-regulated CG-dependent cell death, BM cells were treated with the lysosomotropic agent Leu-Leu-OMe (LLME). LLME accumulates in lysosomes where the acyl transferase activity of DPPI generates hydrophobic (Leu-Leu)_n-OMe polymers that induce lysosomal membrane permeabilization (LMP) and cytotoxicity in granule bearing cells such as cytotoxic T-lymphocytes, NK cells and myeloid cells^{29,30}. While the cytotoxic effect of LLME and other methyl ester derivatives on PMNs has long been described, the contribution of PMN granule serine proteases to this process following LMP is unknown. First, we confirmed that DPPI-deficiency was completely protective against LLME-induced cell death in PMNs (data not shown). Then, we found that LLME significantly decreased the survival of WT PMNs; however, this effect was even more pronounced in *sB1*^{-/-} PMNs compared to WT PMNs (Fig.5A, left panel). In contrast, survival of *CG*^{-/-} and *CG.sB1*^{-/-} PMNs was identical to each other but significantly greater than that of WT PMNs (P<0.001 after 2 and 4 hr) (Fig.5A, middle panel). Finally *NE.sB1*^{-/-} PMNs were more sensitive to LLME compared to *NE*^{-/-} PMNs but deficiency in NE alone had no further effect on PMN survival in the presence of LLME when compared with WT PMN survival (Fig.5A, right panel) and the reduced survival of *NE.sB1*^{-/-} PMNs is similar to that of *sB1*^{-/-} PMNs, reflecting mainly the absence of serpinB1 (Fig.5A, right panel). In the presence of caspase inhibitor Q-VD-OPh, a similar pattern was observed between the genotypes (Fig.5B). Furthermore, Q-VD-OPh failed to improve the survival of *sB1*^{-/-} and *NE.sB1*^{-/-} PMNs, whereas the survival of WT and *NE*^{-/-} PMNs was significantly increased. In mice lacking CG, caspase inhibition had a modest effect on PMN survival (Fig. 5B). These results

suggest that the cytoplasmic release of CG upon LMP likely initiates cell death upstream and independent of caspases and this effect is especially evident when CG activity is unopposed in the absence of serpinB1.

G-CSF therapy increases *sB1*^{-/-} PMN numbers via enhanced granulopoiesis

G-CSF therapy is an effective long-term treatment in many cases of severe congenital neutropenia (SCN) and it is also used to prevent chemotherapy-induced febrile neutropenia by enhancing PMN production. In addition, G-CSF delays neutrophil apoptosis by differentially regulating pro- and anti-apoptotic factors¹⁰. To test whether G-CSF could rescue *sB1*^{-/-} PMN survival defect, WT and *sB1*^{-/-} mice were treated with therapeutic doses of G-CSF or saline for five days and BM and blood PMNs were analyzed 24hr after the last injection. Total counts of myelocytes and PMNs were significantly increased in the BM of treated mice compared to their respective untreated genotype controls (Fig.6A,B). The increase in myelocyte numbers was identical in G-CSF treated WT and *sB1*^{-/-} mice, indicating that G-CSF-induced granulopoiesis proceeds normally in *sB1*^{-/-} myeloid progenitors (Fig.6B). However, PMN counts of treated *sB1*^{-/-} mice remained significantly lower than that of WT mice (Fig.6A). More strikingly, blood PMN numbers were increased only in G-CSF treated WT but not *sB1*^{-/-} treated mice. In G-CSF-treated *sB1*^{-/-} mice, blood PMN numbers were identical to the baseline numbers for *sB1*^{-/-} mice (Fig.6C; Suppl Fig.S6). These results indicate that *sB1*^{-/-} PMN precursor proliferation is normal in response to G-CSF therapy leading to expansion of the BM reserve to some extent. However, the lower number of PMNs in the BM and, particularly, the lack of a sustained blood neutrophilia suggest that G-CSF does not fully rescue the survival defect of mature *sB1*^{-/-} PMNs.

Discussion

SerpinB1 is a member of the clade B serpins, a subfamily composed of leaderless proteins with nucleocytoplasmic localization. Clade B serpins are often expressed in cells that also carry target proteases, which led to the hypothesis that intracellular serpins protect against misdirected granule proteases and/or protect bystander cells from released proteases³¹. We previously reported that deficiency in serpinB1 is associated with reduced PMN survival in the BM and at inflammatory sites^{19,20}. The evidence presented here demonstrates that the cytoprotective function of serpinB1 in PMNs is based on the inhibition of granule protease cathepsin G (CG). Deficiency in CG was sufficient to rescue the defect of *sB1*^{-/-} mice as illustrated by normal PMN counts in the BM of double knockout *CG.sB1*^{-/-} mice. We also showed that the

protease-serpin interaction occurred within PMNs. Indeed, WT PMNs had a greater survival over *sB1*^{-/-} PMNs in mixed BM chimera, whereas the survival of *CG.sB1*^{-/-} PMNs was similar to WT PMNs after BM transfer. SerpinB1 is an ancestral clade B serpin with a conserved specificity determining reactive center loop in all vertebrates³². Furthermore, human and mouse serpinB1 have the same specificity for chymotrypsin-like and elastase-like serine proteases^{21,22}. Likewise, human and mouse CG have identical substrate specificities and the phenotype of *CG*^{-/-} murine PMN can be rescued by human CG³³. Therefore, it is highly likely that the antagonistic functions of CG and serpinB1 in cellular homeostasis observed in mice can be extended to other species.

Extracellular CG was previously reported to promote detachment-induced apoptosis (anoikis) in human and mouse cardiomyocytes³⁴. This activity is mediated through the shedding and transactivation of epidermal growth factor receptor (EGFR) and downregulation of focal adhesion signaling^{35,36}. In our study, exogenous human CG also induced PMN death in vitro but these effects were not enhanced in *sB1*^{-/-} PMNs and the neutropenia associated with serpinB1 deficiency was principally cell-intrinsic. How intracellular CG induces PMN death remains to be fully investigated. However, our studies provide some indications on the potential pathways. Like other NSPs, the expression of CG is transcriptionally restricted to the promyelocyte stage during PMN development and NSPs are then stored in active form in primary azurophil granules³⁷. Since serpinB1 is equally efficient at inhibiting NE, CG and PR3, it was surprising that deletion of CG alone was sufficient to achieve a complete reversal of the PMN survival defect in *CG.sB1*^{-/-} mice. A possible explanation would be that CG gains access to targets more readily than other granule proteases. There is evidence that binding to serglycin proteoglycans differs between NE and CG resulting in altered sorting of NE but not CG into granules of serglycin-deficient PMNs³⁸. Different interactions with granule matrix may thus contribute to differential release of CG from the granules compared to other NSPs. However, because *sB1*^{-/-} PMNs have similar levels of CG and NE as WT PMNs²⁰ and because LLME-induced granule permeabilization likely releases all granule contents equally, we favor an alternative interpretation where CG specifically targets essential cellular components that are not cleaved by the other serpinB1-inhibitable granule proteases. Upon granule permeabilization, we found that CG can induce cell death upstream of caspases as well as independent of caspases. CG was previously shown to activate caspase-7 in vitro and it functions at neutral pH, which is consistent with a physiological role in the nucleocytoplasmic environment³⁹. Cell death induced by lysosomal/granule

membrane permeabilization has previously been linked to cysteine cathepsins in other cell types. However, these proteases appear to depend on caspase activation to trigger apoptosis and they function poorly at neutral pH, questioning their potential role as regulators cell death⁴⁰. In contrast, CG-mediated cell death is not completely blocked by caspase inhibition, which is a property reminiscent of granzymes in cytotoxic T cells⁴¹. In fact, CG is phylogenetically most closely related to serine proteases granzyme B and H⁴². Granzymes have numerous nuclear, mitochondrial and cytoplasmic target proteins leading to cell death⁴¹ and we anticipate that this may also be the case for CG.

Whether intracellular release of CG induces death in other cell types remains to be investigated. CG and CG-inhibitory serpins, such as serpinB1 and serpinB6, are also expressed in monocytes, dendritic cells and mast cells and the protease-serpin balance in these cells may be altered in specific stress conditions leading to cell death. Interestingly, human gzmH has a chymotrypsin-like activity similar to CG and was recently shown to be inhibited by serpinB1 in NK cells⁴³. However, mice have many homologs of gzmB and gzmH that have diverged from their human counterparts⁴⁴. Therefore it is yet unclear whether mouse serpinB1 would also inhibit one or several of mouse granzymes. In cancer cells, TNF- α mediated apoptosis has been associated with induction of CG expression and apoptosis was partly prevented by transfection with human squamous cell carcinoma antigen 2 (SCCA2)(serpinB4), a serum marker for advanced squamous cell carcinoma tumors. SCCA2 inhibits CG, although with lower second order rate constants than serpinB1 or serpinB6^{22,45}. These findings, while only associative, are reminiscent of the upregulation of serpinB9 in tumor cells as a protective strategy against CTL death mediated by gzmB and perforin⁴⁶⁻⁴⁸. Whether serpins' inhibition of CG activity in tumors contributes to anti-apoptotic resistance of cancer cells should be interesting to investigate.

G-CSF therapy is successfully used to treat most congenital and acquired neutropenia through increased granulopoiesis, mobilization from the BM and increased survival of PMNs. Prosurvival effects of G-CSF includes the upregulation of anti-apoptotic Bcl-2 family members, which act upstream of the mitochondria and the activation of effector caspases. In *sB1*^{-/-} mice, G-CSF levels in serum are 4-fold higher than in WT mice in steady state and this is accompanied by an upregulation of the anti-apoptotic Bcl-2 family member Mcl-1 in *sB1*^{-/-} PMNs¹⁹. Here, G-CSF therapy significantly increased granulopoiesis in both WT and *sB1*^{-/-} mice. However, the PMN numbers in treated *sB1*^{-/-} BM and blood were significantly lower than those of treated WT mice, indicating only a partial rescue of the survival defect. This is consistent with

our findings that CG-mediated death can proceed independently of caspases and can thus bypass anti-apoptotic effects mediated by G-CSF.

CG has largely been studied in association with antimicrobial and inflammatory functions due to its presence in PMNs^{12-14,49}. In this context, we have previously shown that serpinB1 contributes to prevent increased mortality and morbidity associated with production of inflammatory cytokines upon infection with *Pseudomonas aeruginosa* and influenza A virus^{20,50}. In this study, we demonstrate that serpinB1 inhibition of the primary granule protease CG in PMNs is essential for PMN survival and this ultimately regulates PMN numbers in vivo. Our findings also extend the roles of CG from antimicrobial and immunoregulatory functions to a novel role in inducing cell death.

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Authorship Contributions and Disclosure of Conflicts of Interest

MB performed research, analyzed and interpreted data, performed statistical analysis and drafted the manuscript. C.T.N.P. contributed vital reagents and to writing the manuscript. CB designed research, analyzed and interpreted data and wrote the manuscript.

All authors declare no conflicts of interest.

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Figures and figure legends

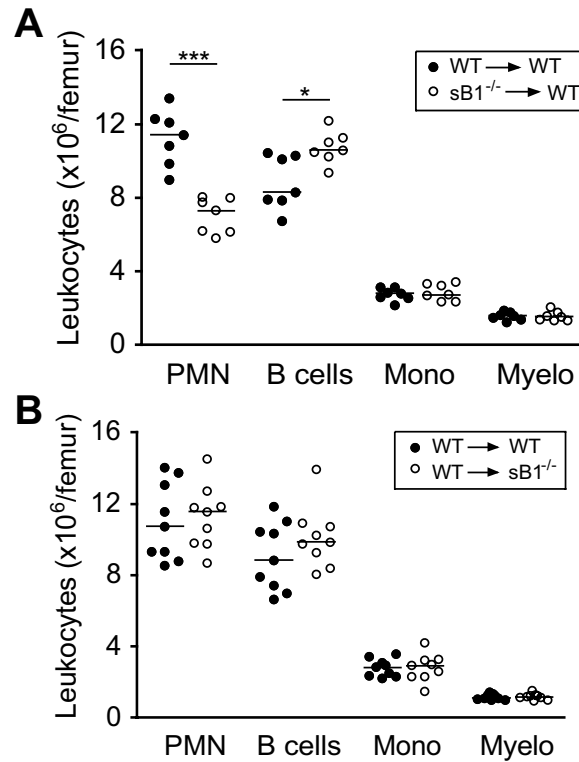


Figure 1. Defective PMN reserve in BM chimera depends on serpinB1-deficiency in the hematopoietic compartment. Flow cytometry analysis of major BM leukocyte subsets of lethally irradiated mice was performed 8-10 weeks after BM transfer. **(A)** Irradiated WT (CD45.1) mice were transferred with WT (filled circles) or *sB1*^{-/-} (open circles) BM cells. **(B)** Irradiated WT (filled circles) and *sB1*^{-/-} (open circles) mice both CD45.2 were transferred with WT (CD45.1) BM cells. Each circle represents leukocyte number for one mouse and horizontal line indicates the median. Median subsets numbers were compared by Mann-Whitney test (* $P < 0.05$; *** $P < 0.001$).

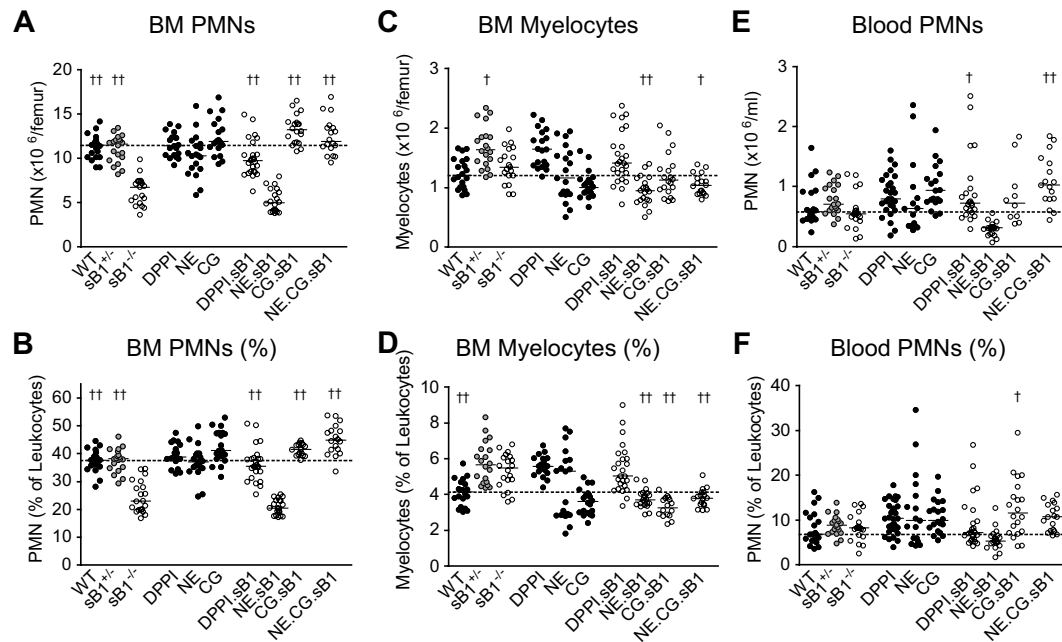


Figure 2. PMN and myelocyte numbers in BM and blood of mice deficient in NSPs and serpinB1. Cell counts and percentages were determined by flow cytometry analysis for BM PMNs (**A,B**), myelocytes (**C,D**) and blood PMNs (**E,F**) in 8 week old mice. Each circle represents the value for one mouse, horizontal bars indicate median value for each genotype (BM: N=19-25; blood: N=18-35). The dotted line across the graph shows the median for WT mice. For each subset, median values of double and triple knockout mice lacking *sB1* and one or several NSPs (open circles) were analyzed by one-way ANOVA followed by Dunnett's comparison relative to reference column for *sB1*^{-/-} mice († P<0.05; †† P<0.01).

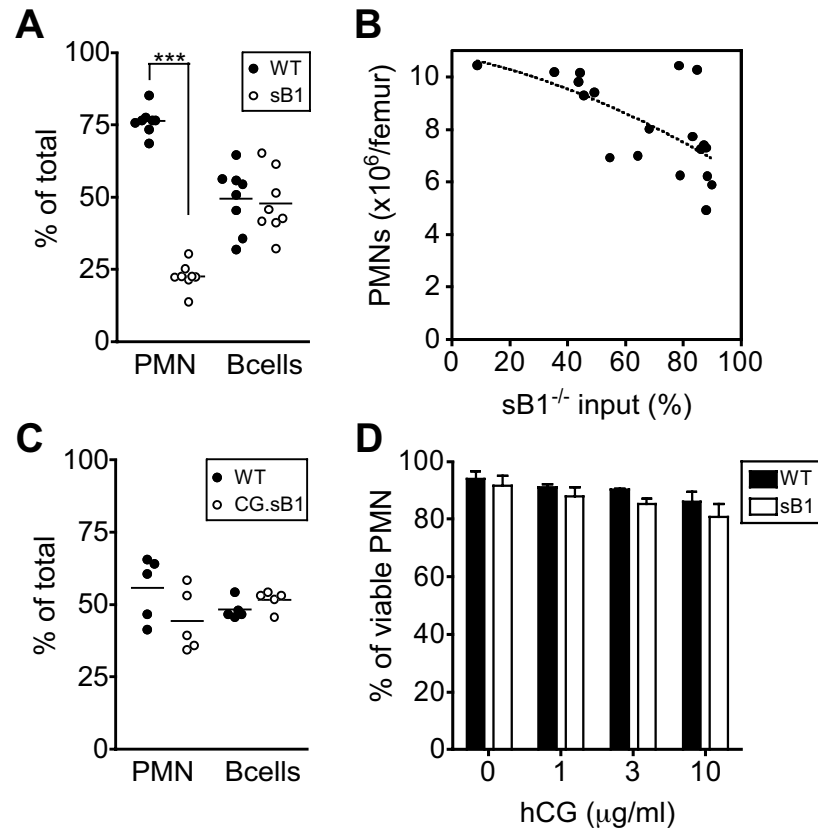


Figure 3. CG-mediated *sB1*^{-/-} PMN death is cell intrinsic. (A) The relative percentage of cells from each donor is shown for PMNs and B cells from irradiated GFP⁺ mice 8-10 weeks after transfer of 1:1 mixture of WT (CD45.1) and *sB1*^{-/-} (CD45.2) BM. Data points are indicated for each mouse and means were compared by paired Student's t-test (***) $P < 0.001$. (B) Total PMN numbers in BM of irradiated GFP⁺ mice are shown 8-10 weeks after transplant with varying amounts (1:1 or 1:4) of BM cells of WT and *sB1*^{-/-} donors relative to the percentage of *sB1*^{-/-} input. The dotted line indicates negative correlation between *sB1*^{-/-} input and total PMN numbers. (C) The relative percentage of cells from each donor is shown for PMNs and B cells from irradiated GFP⁺ mice 8-10 weeks after transfer of 1:1 mixture of WT (CD45.1) and CG.*sB1*^{-/-} (CD45.2) BM. Data points are indicated for each mouse and means were compared by paired Student's t-test. (D) Survival of WT and *sB1*^{-/-} PMNs in vitro in the presence of human CG for 3hr in serum-free medium. Percentage of live cells (mean±SD) of 2-5 independent experiments were compared by two-way ANOVA with Bonferroni post-test.

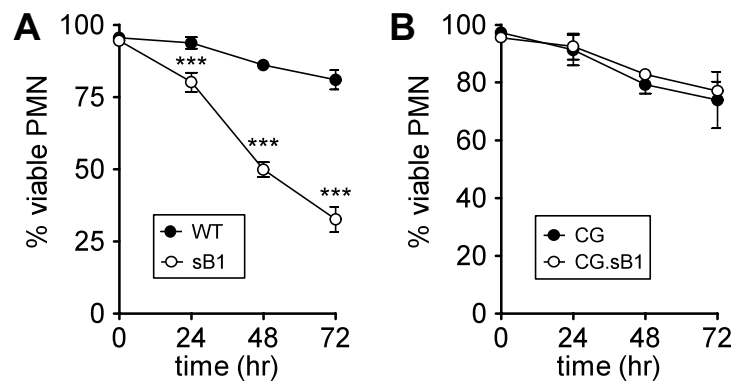


Figure 4. sB1^{-/-} PMN death mediated by CG does not require caspase activity. Survival of isolated BM PMNs in the presence of 50μM pan-caspase inhibitor Q-VD-OPh was measured by flow cytometry (Suppl Fig.S1) at indicated time points for **(A)** WT and sB1^{-/-} PMNs (N=3) and **(B)** CG^{-/-} and CG^{-/-}.sB1^{-/-} PMNs (N=2). Data is shown as mean ± SD and was analyzed by two-way ANOVA followed by Bonferroni post test (***) P<0.001).

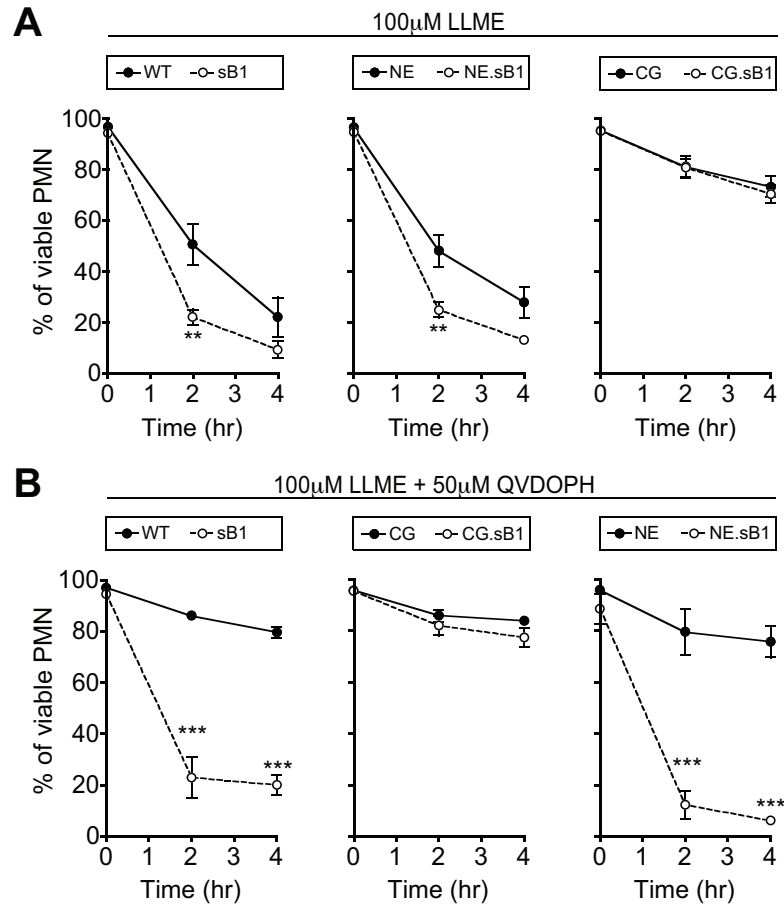


Figure 5. LMP induces CG-mediated death in PMNs. BM cells of indicated genotypes were incubated with 100 μ M of LLME only (**A**) or 100 μ M LLME and 50 μ M Q-VD-OPh (**B**). Percentage of surviving PMNs was determined by flow cytometry. Data is shown as mean \pm SEM (N=3-5) and was analyzed by two-way ANOVA followed by Bonferroni post-test (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

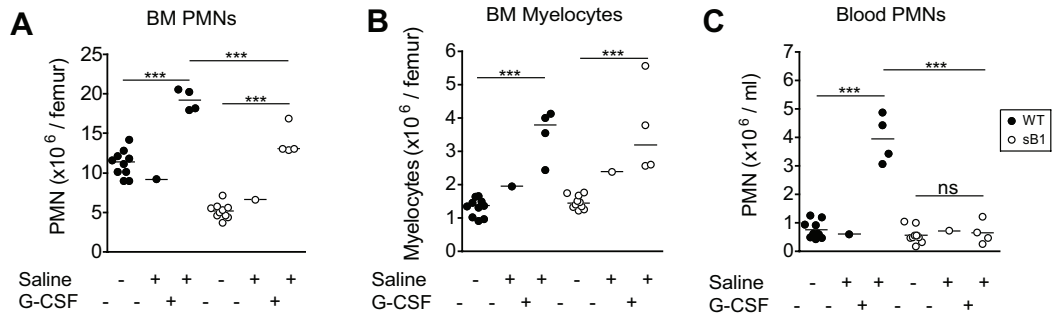


Figure 6. In vivo G-CSF therapy increases PMN numbers in BM of *sB1*^{-/-} mice. BM PMN (A), myelocyte (B) and blood PMN (C) numbers were determined 24hr after recombinant murine G-CSF therapy for 5 consecutive days. Filled and open circles represent cell numbers of individual WT and *sB1*^{-/-} female mice, respectively. Untreated females (Fig.2 subset) were similar to saline control females and were compared to the G-CSF treated mice. Statistical analysis was performed using two-way ANOVA with Bonferroni post test. (** P<0.01; *** P<0.001).